

An Amplified Fragment Length Polymorphism Map of the Silkworm

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ABSTRACT

The silkworm (*Bombyx mori* L.) is a lepidopteran insect with a long history of significant agricultural value. We have constructed the first amplified fragment length polymorphism (AFLP) genetic linkage map of the silkworm *B. mori* at a LOD score of 2.5. The mapping AFLP markers were genotyped in 47 progeny from a backcross population of the cross no. 782 × od100. A total of 1248 (60.7%) polymorphic AFLP markers were detected with 35 *Pst*I/*Taq*I primer combinations. Each of the primer combinations generated an average of 35.7 polymorphic AFLP markers. A total of 545 (44%) polymorphic markers are consistent with the expected segregation ratio of 1:1 at the significance level of $P = 0.05$. Of the 545 polymorphic markers, 356 were assigned to 30 linkage groups. The number of markers on linkage groups ranged from 4 to 36. There were 21 major linkage groups with 7–36 markers and 9 relatively small linkage groups with 4–6 markers. The 30 linkage groups varied in length from 37.4 to 691.0 cM. The total length of this AFLP linkage map was 6512 cM. Genetic distances between two neighboring markers on the same linkage group ranged from 0.2 to 47 cM with an average of 18.2 cM. The sex-linked gene *od* was located between the markers P1T3B40 and P3T3B27 at the end of group 3, indicating that AFLP linkage group 3 was the Z (sex) chromosome. This work provides an essential basic map for constructing a denser linkage map and for mapping genes underlying agronomically important traits in the silkworm *B. mori* L.

LINKAGE maps have become powerful research tools in genetic studies of many organisms (*e.g.*, WADA *et al.* 1995; KNAPIK *et al.* 1996; DIB *et al.* 1997; DIETRICH *et al.* 1997). A complete linkage map is necessary to efficiently carry out molecular-based analyses such as molecular marker-assisted selection (CHO *et al.* 1994; RÖDER *et al.* 1998), quantitative trait loci (QTL) mapping of agronomically important traits (LANDER and BOTSTEIN 1989; RÖDER *et al.* 1998), prediction of heterosis, and comprehensive investigations of genomic evolution between lineages (MORIZOT *et al.* 1977; MORIZOT 1983; LYONS *et al.* 1997).

Although there has been steady progress in constructing molecular linkage maps, many available techniques are limited for various reasons. Gene mapping employing restriction fragment length polymorphisms (RFLPs) is often limited because of the relatively low level of RFLPs in many organisms, such as wheat (CHAO *et al.* 1989; KAM-MORGAN *et al.* 1989; LIU *et al.* 1990; CADALEN *et al.* 1997) and the silkworm (SHI *et al.* 1995), and thus requires the use of populations derived from wide crosses (RÖDER *et al.* 1998). However, in an intra-specific context, QTL mapping of many agronomically

important traits requires more informative markers than RFLP. This is especially the case for marker-assisted selection (RÖDER *et al.* 1998).

Recently, the amplified fragment length polymorphism (AFLP) technique (Vos *et al.* 1995) has proved to be a convenient and reliable tool to generate highly polymorphic molecular markers that greatly facilitate building linkage maps (BECKER *et al.* 1995; QI *et al.* 1997; WAUGH *et al.* 1997). AFLP is a PCR-based technique that avoids the laborious steps involved in RFLP mapping and generally shows a much higher level of polymorphism and informativeness than any other marker system in the organisms examined so far (MACKILL *et al.* 1996). In the AFLP technique, dozens of restriction fragments (~50–100) from a total digest of genomic DNA are amplified in PCR by using oligonucleotide adaptor and restriction site DNA sequences as target sites for primer annealing (Vos *et al.* 1995). The main value of this technique is that a very large array of polymorphisms is efficiently and quickly sampled, although each individual polymorphism may not be as highly informative as microsatellite markers.

Since their relatively recent discovery, AFLP markers have been widely employed to construct linkage maps of a variety of agronomically important organisms, such as barley (BECKER *et al.* 1995; CASTIGLIONI *et al.* 1998), melon (*Cucumis melo* L.; WANG *et al.* 1997), the oomycete

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(*Phytophthora infestans*; VAN DER LEE *et al.* 1997), soybean (Gycine; KEIM *et al.* 1997), chicken (*Gallus domesticus*; HERBERGS *et al.* 1999; KNORR *et al.* 1999), and rice (*Oryza sativa*; ZHU *et al.* 1999). AFLP markers have also been successfully employed to map agronomically important genes such as the cyst nematode resistance locus *Gpa2* in potato (ROUPPE VAN DER VOORT *et al.* 1997), the *Mlo* gene in barley (SIMONS *et al.* 1997), the *Rx* gene for extreme resistance to potato virus X in tetraploid potato (BENDAHMANE *et al.* 1997), and QTL for resistance to *Bacillus thuringiensis* toxins in the diamondback moth (HECKEL *et al.* 1999).

The silkworm (*Bombyx mori* L.) is an important insect with a long history of great agronomic value. Understanding its genome organization using molecular markers is important for genetic studies and for breeding purposes. PROMBOON *et al.* (1995) constructed a linkage map for the silkworm with 169 markers using randomly amplified polymorphic DNA (RAPD) and YASUKOCHI (1998) built a dense RAPD linkage map, containing 1018 genetic markers on all the 27 autosomes and the Z (sex) chromosome. Although RAPD is a convenient tool to generate polymorphic markers in general, the polymorphic level of RAPD markers is much lower than that of AFLP markers (SHARMA *et al.* 1996). However, a linkage map of the silkworm constructed using AFLP markers has never been reported. In addition, the RAPD linkage maps were constructed employing an F_2 population, which may not be appropriate for dominant type markers such as RAPD and AFLP, since recombination occurs only in males in the silkworm. Our purpose here is to build the first AFLP-based linkage map of the silkworm using a backcross population.

MATERIALS AND METHODS

Insect material: The silkworm (*B. mori* L.) strains employed were no. 782 and od100. No. 782 is a bivoltine variety from Japan, whose body color in the larval stage is white. od100 is a Chinese multivoltine variety, whose larval body color is distinct oily translucence, which is controlled by a recessive mutation, *od*, on the Z (sex) chromosome. The variety no. 782 has a dominant allele of the *od* gene (wild type). Genetic recombination occurs only in males in *B. mori* and other lepidopteran insects. This Lepidoptera-related phenomenon leads to the fact that marker loci derived from the female and male parents cannot be integrated into a single map using an F_2 population. Because of this, our mapping was performed on a backcross population. An F_1 male moth of the cross no. 782 \times od100 was crossed to a female moth of the recurrent parent od100 to generate a BC1 population. The crossing experiments were performed in the Institute of the Silkworm and Mulberry, Southwest Agricultural University of the People's Republic of China. Mapping was performed with 47 silkworm pupae after anchoring the gene *od* in the backcross population.

AFLP analysis: Genomic DNA of individual silkworms was extracted from pupae as described by BENDER *et al.* (1983) and by PROMBOON *et al.* (1995). It was purified by extraction with phenol/chloroform, precipitated by ethanol, and resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE) buffer.

The original AFLP procedure as described by ZABEAU and Vos (1993) and Vos *et al.* (1995) was followed using a minor modification as follows. The restriction enzymes used were *Pst*I and *Taq*I, which produce polymorphic DNA fragments in the silkworm. Adapters used were as follows:

| CORE | ENZ |
|----------------------|----------|
| 5'-GACGTGACGGCCGTC | ATGCA-3' |
| 3'-GCACTGCCGGCAG | T-5' |
| <i>Pst</i> I adapter | |

| CORE | ENZ |
|----------------------|--------|
| 5'-GACGATGAGTCCTGA | G-3' |
| 3'-TACTCAGGACT | CGC 5' |
| <i>Taq</i> I adapter | |

The primers corresponding to the *Pst*I adapter and *Taq*I adapter were as follows:

| CORE | ENZ | EXT |
|--|-------|--------|
| Primer- <i>Pst</i> I-1, 5' GACGGCCGTC | TGCAG | A 3' |
| Primer- <i>Taq</i> I-1, 5' GATGAGTCCTGAG | CGA | A 3' |
| Primer- <i>Pst</i> I-2, 5' GACGGCCGTC | TGCAG | NNN 3' |
| Primer- <i>Taq</i> I-2, 5' GATGAGTCCTGAG | CGA | NNN 3' |

The primer-*Pst*I-1 and primer-*Taq*I-1 were used for the pre-amplification reaction. The 10- μ l PCR preamplification reaction system contained 2.5 ng template DNA, 30 ng of both primer-*Pst*I-1 and primer-*Taq*I-1, 0.2 units Taq polymerase, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), and 0.1% Triton X-100. The preamplification reaction conditions and steps employed were as described by Vos *et al.* (1995). After the preamplification reaction, the 10- μ l PCR amplification reaction system contained 1 μ l product of the diluted preamplification reaction, 30 ng primer-*Pst*I-2, 15 ng primer-*Taq*I-2, 0.2 units Taq polymerase, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), and 0.1% Triton X-100. The amplification reaction conditions and steps were as described by Vos *et al.* (1995). All amplification reactions were performed in a PTC200 (Gene Company). The primer-*Pst*I-2 and primer-*Taq*I-2 used in the second amplification reactions are listed in Table 1. There were 35 primer combinations.

Gel analysis: The products of the second amplification reaction were detected by silver staining on a large denaturing polyacrylamide gel (Promega, Madison, WI) as described by CHALHOUB *et al.* (1997) and by Q4132 silver stain kits for sequencing (Promega) containing 500 μ l bind silane, 10 \times 2 g AgNO₃ (Promega), 20 \times 3 ml 37% formalin (formaldehyde), 10 \times 60 g Na₂CO₃ (Promega), and 10 \times 1 ml 100 mg/ml sodium thiosulphate.

Linkage analysis and map construction: We performed χ^2 -tests for each of the AFLP bands generated to determine whether the segregation ratio of presence/absence in BC1 was significantly different from the expected ratio of 1:1. We eliminated the AFLP data showing an unexpected segregation ratio at the $P = 0.05$ level of significance. A data matrix was constructed from the presence/absence of all polymorphic bands for all 47 backcrossed individuals. This data matrix was input into the program package MAPMAKER/EXP (version 3.0; LANDER *et al.* 1987). The "GROUP" command (LOD 3.0, maximum recombination fraction 0.3) was used to segregate all informative markers into linkage groups. The "SUGGEST SUBSET" command was employed to find highly informative and well-spaced markers within each group. The framework map was constructed using some markers as a subsequence

TABLE 1
The primers used in the second amplification

| Primer- <i>Pst</i> I-2 | Primer- <i>Taq</i> I-2 |
|------------------------------|-------------------------------|
| P1: 5'-GACGGCCGTATGCAGAAT-3' | T1: 5'-GATGAGTCCTGAGCGAAAT-3' |
| P2: 5'-GACGGCCGTATGCAGAAG-3' | T2: 5'-GATGAGTCCTGAGCGAAAC-3' |
| P3: 5'-GACGGCCGTATGCAGAAC-3' | T3: 5'-GATGAGTCCTGAGCGAACA-3' |
| P4: 5'-GACGGCCGTATGCAGATA-3' | T4: 5'-GATGAGTCCTGAGCGAAAG-3' |
| P5: 5'-GACGGCCGTATGCAGATC-3' | T5: 5'-GATGAGTCCTGAGCGAAGA-3' |
| P6: 5'-GACGGCCGTATGCAGATG-3' | T6: 5'-GATGAGTCCTGAGCGAAGC-3' |
| P7: 5'-GACGGCCGTATGCAGAGA-3' | |

within each of the major linkage groups. The "ORDER" and the "COMPARE" commands were used to identify the most probable marker order within a linkage group. The order was verified using the "RIPPLE" command. Markers were retained within the framework of this map only if the LOD value for RIPPLE was at least 2.5. The "TRY" command was used to assign additional markers to intervals within the LOD 2.5 framework, followed by the process of submitting marker orders to COMPARE and reconfirming the framework of this map with a LOD > 2.5 using the RIPPLE command. Typing errors were detected with the ERROR DETECTION option and the map distance between neighboring markers was calculated according to the Kosambi function (KOSAMBI 1944).

RESULTS

The AFLP linkage map: A total of 2056 AFLP bands were clearly detected with the 35 *Pst*I/*Taq*I primer combinations, of which 1248 bands were polymorphic. Those bands that were not readable enough were not employed in this study. The frequency of polymorphic AFLP markers from the clearly detected bands in the no. 782 × od100 cross in the silkworm was 60.7%, significantly higher than that (14.0%) in the Proctor × Nudinka cross reported in barley (CASTIGLIONI *et al.* 1998). Each primer combination generated an average of 35.7 polymorphic markers. A total of 545 polymorphic markers fell in the expected segregation ratio of 1:1 at the $P = 0.05$ level. About 56% polymorphic markers showed deviation from the expected 1:1 ratio of the $P = 0.05$ significance level. A total of 356 of the 545 markers were assigned to 30 linkage groups. The number of markers on linkage groups ranged from 4 to 36. There were 21 major linkage groups with 7 to 36 markers and 9 relatively small linkage groups with 4 to 6 markers in this AFLP linkage map (Figure 1). Linkage groups ranged in length from 37.4 to 691.0 cM. The total length for this AFLP linkage map was 6512 cM. The distances between two neighboring markers ranged from 0.2 to 47.5 cM with an average of 18.24 cM. The *od* gene on the Z chromosome of the classical map (XIANG 1995) was located between the markers P1T3B40 and P3T3B27 on one end of group 3. Hence group 3 was probably the Z chromosome.

After typing errors were detected with the MAP-MAKER ERROR DETECTION option, corrections in

the total length of the map led to a reduction from 6807.6 to 6512.0 cM. Relatively larger changes were observed in the major linkage groups. Groups 1, 2, 4, and 5 were shortened by 20.5, 13.5, 18.3, and 18.5%, respectively. Other linkage groups were shortened by no more than 10%. The reduction (4.33%) of our map was, in total length, much less than that (40.3%) of the AFLP map of barley reported by CASTIGLIONI *et al.* (1998).

The distributions of the mapping AFLP markers among the primer combinations: Data in Table 2 provide information on the distribution of mapping AFLP markers among the 35 primer combinations for the cross of no. 782 × od100. The proportion of markers employed for mapping to those consistent with the expected segregation ratio of 1:1 was, on average, 62.0%. For most primer combinations, ~10–15 markers is useful for mapping; only P1T1, P3T5, P4T1, P4T5, and P6T2 generated poorly informative markers for mapping, and the distributions of mapping markers among their primer combinations ranged from 5 to 19. Only one primer combination, P7T3, did not produce any informative marker for mapping. Overall, primers P1, P2, P3, P5, T3, T4, and T6 were more efficient in generating informative markers than P4, P6, T1, T2, and T5 in these two strains. These data showed that the AFLP technique produces highly informative markers for gene mapping in the silkworm. We did not find that markers from any primer combination were clustered on only one linkage group or in only one region.

DISCUSSION

A total of 61% of AFLP bands that were clearly readable and thus evaluated were polymorphic between the no. 782 and od100 strains of the silkworm. This high level of polymorphism between the two strains may be due to the fact that only bands representing relatively small fragments that were well scattered and readable from the middle to the bottom of the gels were counted. About 60% of the total bands obtained in the genotyping efforts were monomorphic and/or relatively large. Because of the low resolution from the middle to the

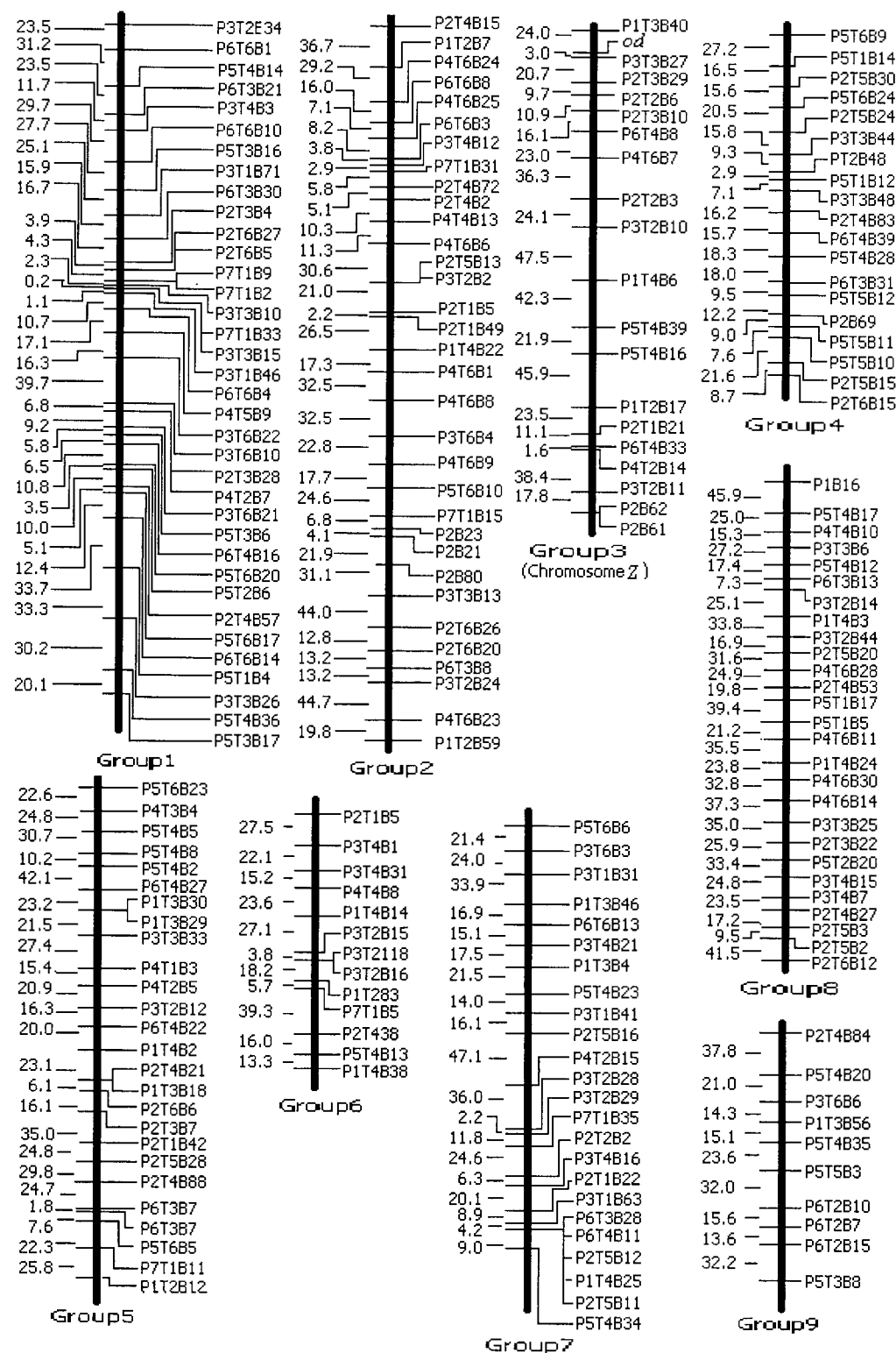


FIGURE 1.—AFLP genetic linkage map of the silkworm based on 47 backcrossed progeny derived from the no. 782 × od100 cross. The markers are represented by *Pst*I primer number, *Taq*I primer number, and band number. The *od* gene on the Z chromosome of the classical map of the silkworm is located on group 3. The numbers on the left-hand side of each linkage group are the genetic distances in each interval measured in centimorgans.

top of the gels, these large bands were not counted in our mapping research. About 40% of the total bands were polymorphic. About 56% of the polymorphic markers showed deviation from the expected 1:1 segregation ratio at the $P = 0.05$ significance level. Hence, 22.4% of all the markers deviated from the expected 1:1 ratio.

The high level of polymorphic AFLP markers in those bands counted may be due to several factors. First, no. 782 and od100 are two distinct silkworm strains. The former was from the Japanese bivoltine system and the latter was from the Chinese multivoltine system. A high level of DNA polymorphisms is not unexpected between the two systems. Second, AFLP is a tool for detecting

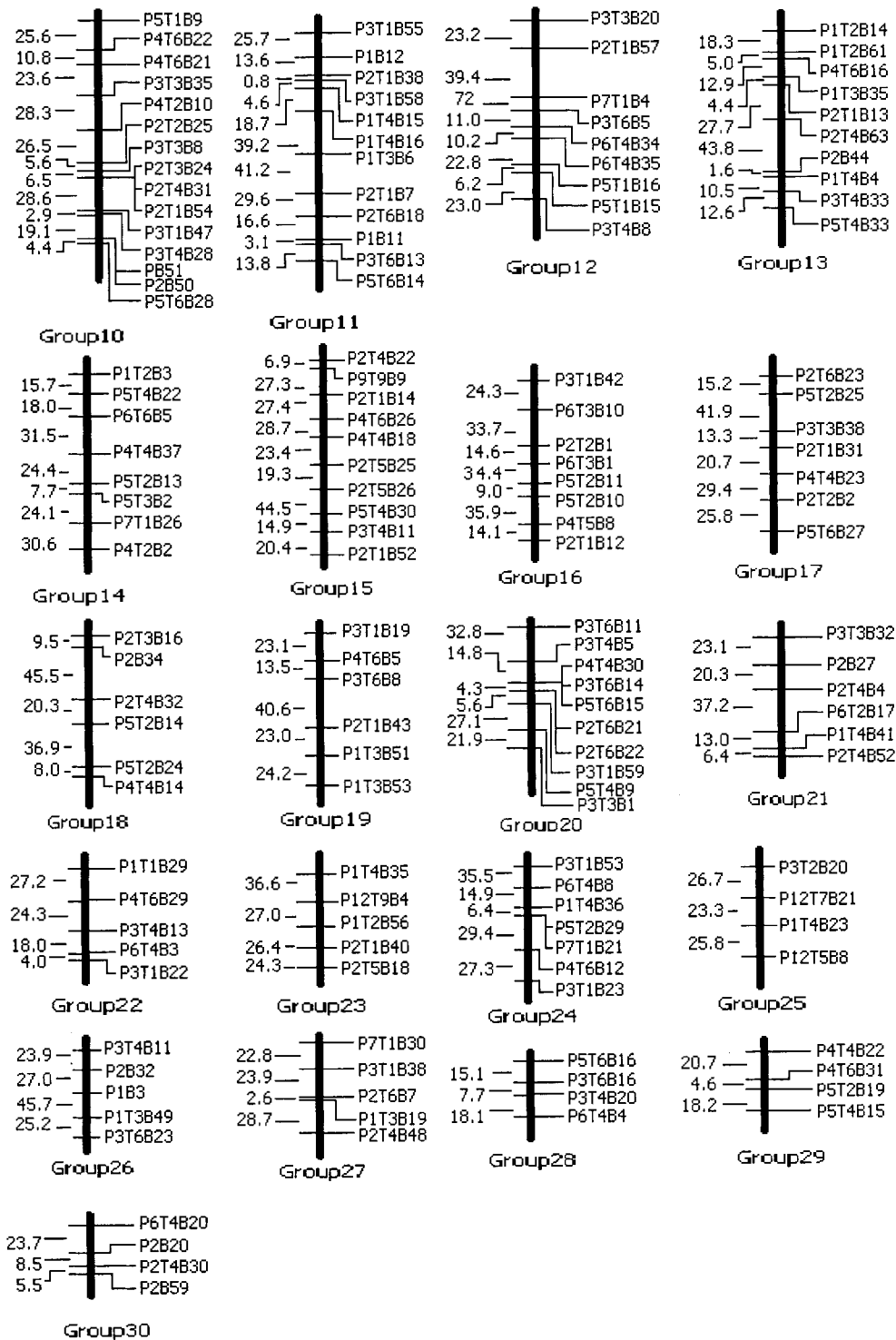


FIGURE 1.—Continued.

high levels of polymorphisms (HUYS *et al.* 1996; LATORRA *et al.* 1996; MACKILL *et al.* 1996; WAN *et al.* 1999). Dozens of restriction fragments (~50–100) from a total digest of genomic DNA are amplified in PCR by using oligonucleotide adaptor and restriction site DNA sequences as target sites for primer annealing (Vos *et al.* 1995). Third, a large fraction of the silkworm genome consists of families of transposable elements such as *Bm1*, *BMC1* (a member of the *LINE1* family), *mariner*, *mariner*-like

elements (*Bmmar1*), long terminal repeat transposons (LTRs), nonlong terminal transposons (nonLTRs), and so on (UEDA *et al.* 1986; HERRER and WANG 1991; XIONG and EICKBUSH 1993; XIONG *et al.* 1993; ROBERTSON and ASPLUND 1996; TOMITA *et al.* 1997; SHIMIZU *et al.* 2000; WANG *et al.* 2000). The chromosomal distribution of these transposable elements has been shown to vary between silkworm strains. If some sites of restriction enzyme cleavage and sequences bound by the primer

TABLE 2
The distribution of the AFLP mapping markers among primer combinations

| Primer combinations | No. of markers ^a (%) | Primer combinations | No. of markers ^a (%) | Primer combinations | No. of markers ^a (%) |
|---------------------|---------------------------------|---------------------|---------------------------------|---------------------|---------------------------------|
| P1 | 4/6 (66.7) | P3T1 | 15/21 (71.4) | P5T1 | 9/12 (75.0) |
| P2 | 14/23 (60.8) | P3T2 | 15/22 (68.2) | P5T2 | 12/16 (75.0) |
| P1T1 | 1/5 (20.0) | P3T3 | 15/22 (68.2) | P5T3 | 7/11 (63.6) |
| P1T2 | 10/11 (90.9) | P3T4 | 16/26 (61.5) | P5T4 | 19/29 (65.5) |
| P1T3 | 12/15 (80.0) | P3T5 | 1/3 (33.3) | P5T5 | 5/9 (55.6) |
| P1T4 | 15/21 (71.4) | P3T6 | 13/17 (76.5) | P5T6 | 15/21 (71.4) |
| P2T1 | 17/25 (68.0) | P4T1 | 1/1 (100) | P6T2 | 3/9 (33.3) |
| P2T2 | 6/15 (40.0) | P4T2 | 5/6 (83.3) | P6T3 | 10/12 (83.3) |
| P2T3 | 8/14 (57.1) | P4T4 | 9/17 (52.9) | P6T4 | 13/18 (72.2) |
| P2T4 | 19/30 (63.3) | P4T5 | 2/6 (33.3) | P6T6 | 7/12 (58.3) |
| P2T5 | 15/20 (75.0) | P4T6 | 19/28 (67.8) | P7T1 | 12/16 (75.0) |
| P2T6 | 12/18 (66.7) | | | P7T3 | 0/8 (0.0) |

Percentage of mapping markers per primer combination: 63.5.

^a The number to the left of the slash is the number of mapping markers; the number to the right of the slash is the number of markers consistent with the expected segregation ratio of 1:1 at $P = 0.05$ significance level; the number in parentheses is the proportion of the less to the more.

combinations distribute on such mobile DNA sequences in the silkworm genome, they could account for some of the polymorphic AFLP markers that were detected. Therefore, a high degree of polymorphism between the no. 782 and od100 is not too surprising.

Deviations from Mendelian segregation ratios have been seen in previous efforts to construct genetic linkage maps using molecular markers (see FARIS *et al.* 1998); 26% of RFLP markers showed distorted segregation ratios in the potato (GEBHARDT *et al.* 1989), and the corresponding number is 44% in barley (GRANER *et al.* 1991). For the AFLP markers, distorted segregation ratios were seen 65% of the time in the clubroot (VOORIPS *et al.* 1997) and 54% of the time in the silkworm as reported here. Distorted segregation of molecular markers may result from competition among gametes for preferential fertilization (LYTTLE 1991), sampling in finite mapping populations, breaking of DNA chains during extraction of DNA samples from tissues, or amplification of a single-sized fragment derived from several different regions (FARIS *et al.* 1998). Markers amplified from near centromeric regions of chromosomes also tend to have distorted segregation ratios (FARIS *et al.* 1998). As mentioned earlier, some markers may have been amplified from transposable elements and such markers may not follow Mendelian segregation ratios.

About 30% of markers showing Mendelian segregation ratios were unlinked in our study. Higher proportions of unlinked markers were observed in mapping studies with other species (GEBHARDT *et al.* 1989; AL-JANABI *et al.* 1993; GRATAGLIA and SEDEROFF 1994; HE 1998). In our results for dominant-recessive AFLP markers, all the unlinked markers detected come from the nonrecurring parent, *i.e.*, no. 782. Therefore, many markers did not show linkage due to the absence of

neighboring markers in the recurrent parent—a consequence of a low density of markers in the region. However, these markers may be used together with markers obtained from other AFLP, RFLP, RAPD, and microsatellite analyses to obtain a denser AFLP map of the silkworm.

The haploid genome of the silkworm has 28 chromosomes and 28 conventional linkage groups have been recognized (DOIRA 1992). Our AFLP map is composed of 30 linkage groups, of which some linkage groups are from the same chromosome. Development of a denser collection of markers may be able to link some of these linkage groups together. Nonequivalence between the number of linkage groups and the number of chromosomes has also been reported in other studies (PROMBOON *et al.* 1995; HE 1998; YOUNG *et al.* 1998). YASUKOCHI (1998) indicated that the large number of chromosomes in the haploid silkworm genome ($n = 28$), typical of lepidoptera, makes it difficult to construct maps without missing some chromosomes.

Analysis of distributions of informative markers among primer combinations indicated that neither all primers nor all primer combinations could produce informative polymorphic markers for mapping. This suggests that, similar to the RAPD technique, AFLP analysis of the whole genome such as we performed here is required to search for primer combinations with highly informative markers. In an F_2 population of the Proctor \times Nudinka cross in barley, on average, a primer combination amplified 87.5 markers, the frequency of AFLP polymorphic markers was 14.0%, and the proportion of informative markers for mapping to polymorphic ones was 61.3% (CASTIGLIONI *et al.* 1998). In comparison, the frequency of AFLP markers that were polymorphic in the backcross in the silkworm was 60.7%. On average, 58.7 markers

per primer combination were amplified and the proportion of informative markers for mapping to polymorphic ones was 63.5%. Therefore, the frequency of polymorphic AFLP markers in the silkworm was much higher than in barley, but the proportion of informative markers for mapping to all the polymorphic ones was similar in both species.

Our AFLP map with an average of 18.24 cM per interval was not particularly dense and had several gaps >30 cM. The relatively large gaps led to the increased length of our map and need to be filled in with additional markers. The distance between neighboring markers on a map is not strongly related to a mapping population size but is determined mainly by the likelihood values of their order when using MAPMAKER/EXP (version 3.0; Y. D. TAN, C. L. WAN and Y. F. ZHU, unpublished results). In addition, typing errors may also be partially responsible for map expansion (LINCOLN and LANDER 1992). CASTIGLIONI *et al.* (1998) reported that use of the ERROR DETECTION option of MAPMAKER reduced the map length from 2673 to 1597 cM, shortening it by 40.3%. After typing errors were detected using the ERROR DETECTION option, our map length was shortened by only 4.33% and we did not find the "inversion" of marker order reported in CASTIGLIONI *et al.* (1998). This result suggests that there were only a few typing errors in our study, which led to the slight increase in length of our map. TAN and MA (1998) demonstrated theoretically that with additional markers typed the map length may increase when marker density is not saturated or may decrease when marker density is in a saturation state. For example, CAUSSE *et al.* (1994) constructed a rice map with 762 markers covering 4026.3 cM, whereas HARUSHIMA *et al.* (1998) obtained a 2275-marker genetic map of rice covering 1521.6 cM. This may explain why the length of our AFLP map is more than that of YASUKOCHI's (1998) RAPD map.

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